

Capsular Polysaccharide of Group A *Streptococcus*

MICHAEL R. WESSELS

Division of Infectious Diseases, Boston Children's Hospital, Harvard Medical School, Boston, MA

ABSTRACT Most clinical isolates of *Streptococcus pyogenes* elaborate a capsular polysaccharide, which is composed of hyaluronic acid, a high-molecular-mass polymer of alternating residues of *N*-acetyl glucosamine and glucuronic acid. Certain strains, particularly those of the M18 serotype, produce abundant amounts of capsule, resulting in formation of large, wet-appearing, translucent or "mucoid" colonies on solid media, whereas strains of M-types 4 and 22 produce none. Studies of acapsular mutant strains have provided evidence that the capsule enhances virulence in animal models of infection, an effect attributable, at least in part, to resistance to complement-mediated opsonophagocytic killing by leukocytes. The presence of the hyaluronic acid capsule may mask adhesins on the bacterial cell wall. However, the capsule itself can mediate bacterial attachment to host cells by binding to the hyaluronic-acid binding protein, CD44. Furthermore, binding of the *S. pyogenes* capsule to CD44 on host epithelial cells can trigger signaling events that disrupt cell-cell junctions and facilitate bacterial invasion into deep tissues. This article summarizes the biochemistry, genetics, regulation, and role in pathogenesis of this important virulence determinant.

INTRODUCTION

In her early studies of group A streptococci (*Streptococcus pyogenes*), Rebecca Lancefield noted an association between virulence and a distinctive appearance of the bacterial colonies on solid media. Isolates that were highly virulent for mice and that grew well in fresh human blood typically formed large colonies with a translucent, liquid appearance (mucoid) or an irregular, collapsed appearance (matte). By contrast, avirulent isolates that grew poorly in human blood formed compact, opaque colonies (glossy). Strains that grew as mucoid or matte colonies usually produced large amounts of M protein, which Lancefield gave the designation "M" because of this association with colony morphology (1, 2). Later work by Armine Wilson demonstrated that the mucoid or matte appearance of

such strains was in fact due to elaboration of capsular polysaccharide, not M protein (3). Wilson showed that the mucoid or matte colony type was converted to a nonmucoid or glossy colony by growth on medium containing hyaluronidase which digested the hyaluronic acid capsule, whereas growth on medium containing trypsin, which digested M protein, had no such effect. Furthermore, while many strains that produced abundant capsular polysaccharide also were rich in M protein, expression of the two surface products was not always linked; certain mucoid or highly encapsulated strains produced little or no M protein, and certain strains rich in M protein produced little or no capsule (1, 4).

Both M protein and capsule have since been shown to contribute independently to *S. pyogenes* virulence. The association of mucoid strains of *S. pyogenes* with both invasive infection and acute rheumatic fever suggested a role for the capsular polysaccharide in viru-

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Correspondence: Michael R. Wessels, michael.wessels@childrens.harvard.edu

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lence. Anecdotal observations that linked the capsule to pathogenesis of streptococcal disease were supported by a study that characterized the colony morphology of more than 1,000 *S. pyogenes* clinical isolates received by a streptococcal reference lab in the 1980s. In that study, only 3% of *S. pyogenes* isolates from patients with pharyngitis had a mucoid colony morphology, whereas 21% of isolates from patients with invasive disease syndromes such as bacteremia, necrotizing fasciitis, or streptococcal toxic shock syndrome and 42% of isolates associated with acute rheumatic fever were mucoid (5). The overrepresentation of mucoid strains among isolates associated with invasive infection and rheumatic fever suggested that the capsule contributes to enhanced virulence. The occurrence of outbreaks of acute rheumatic fever associated with mucoid strains of *S. pyogenes* at several locations in the United States in the 1980s supported the same inference (6–8). These clinical and epidemiologic observations implicating the capsule in pathogenesis have been corroborated by extensive experimental studies that have demonstrated a definite role of the hyaluronic acid capsule as a virulence factor in *S. pyogenes* infection.

BIOCHEMISTRY AND GENETICS OF CAPSULE PRODUCTION

Hyaluronic Acid Biosynthesis

The *S. pyogenes* capsular polysaccharide is composed of hyaluronic acid, a high- M_r ($>10^6$ kDa) linear polymer made up of $\beta(1\rightarrow4)$ -linked disaccharide repeat units of D-glucuronic acid(1 \rightarrow 3)- β -D-N-acetylglucosamine. The polysaccharide is synthesized from the nucleotide sugar precursors UDP-glucuronic acid and UDP-N-acetylglucosamine by a membrane-associated enzyme, hyaluronan synthase. High- M_r hyaluronic acid (or hyaluronan) can be produced by incubation of cell-free membrane extracts of *S. pyogenes* with the two substrate UDP-sugars in the presence of divalent cations (9–11).

A genetic locus required for hyaluronic acid production in *S. pyogenes* was located independently by three laboratories using transposon mutagenesis to produce acapsular mutants from a mucoid *S. pyogenes* strain. Transposon insertions that produced the acapsular phenotype were mapped to a locus that was highly conserved among *S. pyogenes* strains (12–14). Characterization of this region of the *S. pyogenes* chromosome revealed a cluster of three genes, *hasABC*, whose products are involved in hyaluronic acid biosynthesis. The first gene, *hasA*, encodes hyaluronan synthase, a protein with a predicted M_r of 47.9 kDa that includes at least four

predicted membrane-spanning domains, consistent with evidence that the enzyme is membrane associated (15, 16). The *hasA* gene product shares significant similarity with the NodC protein of *Rhizobium meliloti*, which is required for synthesis of an N-acetylglucosamine-containing polysaccharide, with chitin synthases, and with hyaluronan synthases from other microbial and higher animal species (17, 18). The *S. pyogenes* hyaluronan synthase has been expressed in *Escherichia coli* as a His₆-fusion protein. Activity of the purified enzyme is strongly stimulated in the presence of certain phospholipids, particularly cardiolipin (diphosphatidylglycerol), which appears to be associated with the enzyme in *S. pyogenes* (19). Cardiolipin molecules may contribute to formation of a transmembrane pore for export of the hyaluronic acid polymer, since the hyaluronan synthase itself contains only two relatively small extracellular domains (20).

The second gene, *hasB*, encodes UDP-glucose dehydrogenase, a protein with a predicted M_r of 45.5 kDa that catalyzes the oxidation of UDP-glucose to UDP-glucuronic acid (21). The third gene in the cluster, *hasC*, encodes a predicted 33.7-kD protein identified as UDP-glucose pyrophosphorylase (22). The function of this enzyme is to catalyze the condensation of UTP with glucose-1-phosphate to form UDP-glucose. Thus, the reaction catalyzed by the *hasC* product yields a substrate for UDP-glucose dehydrogenase encoded by *hasB*, whose reaction product is, in turn, a substrate for hyaluronan synthase encoded by *hasA* (Fig. 1). While the enzyme protein encoded by *hasC* is enzymatically active, it is not required for hyaluronic acid synthesis by *S. pyogenes*. Selective inactivation of *hasC* resulted in no decrement in hyaluronic acid synthesis by a highly encapsulated strain of *S. pyogenes*; the implication is that another source of UDP-glucose is available within the cell (23). Furthermore, expression of recombinant *hasA* and *hasB* (without *hasC*) resulted in synthesis of hyaluronic acid in *E. coli* and *Enterococcus faecalis* (13, 15). Together, these observations have led to the conclusion that *hasA* and *hasB* are the only two genes uniquely required for bacterial synthesis of hyaluronic acid. The three genes *hasABC* are transcribed as a single message of approximately 4.2 kb from a promoter immediately upstream of *hasA* (24). The identification of a potential *rho*-independent terminator at the 3' terminus of *hasC* suggests that no additional genes are included in the operon. A paralog of *hasB*, *hasB2*, is present at a chromosomal location remote from the *has* operon. Its product is able to support a low level of hyaluronic acid production in the absence of *hasB* (25).

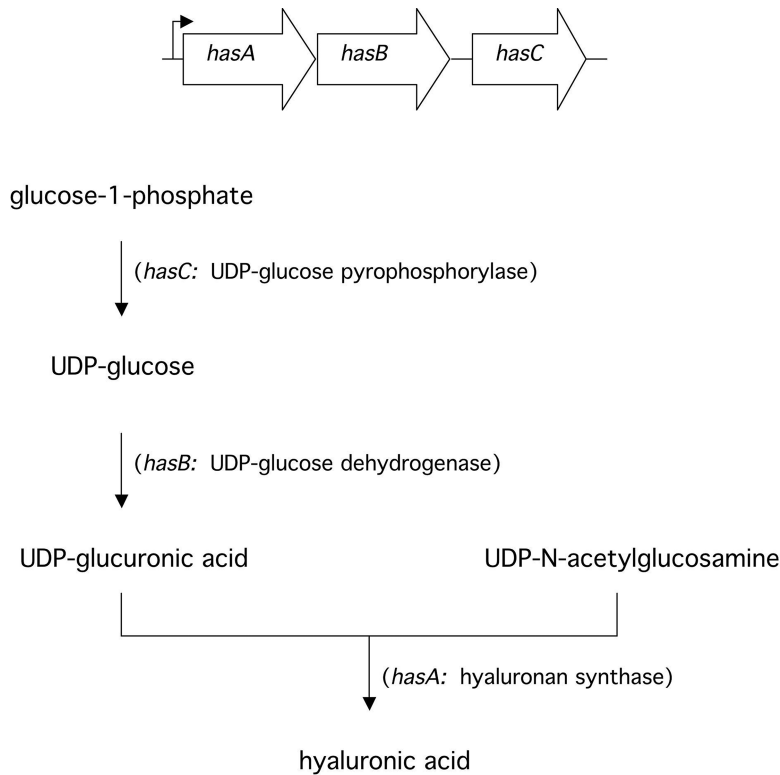


FIGURE 1 Schematic diagram of the *has* operon and the function of each gene product in the biosynthetic pathway for synthesis of hyaluronic acid in *S. pyogenes*.

The simplicity of the capsule gene cluster in *S. pyogenes* contrasts with the size and complexity of capsule synthesis loci in other encapsulated Gram-positive bacteria, which generally consist of at least 12 genes (26–30). One exception is the type 3 pneumococcus, whose capsule production is strikingly similar to that in *S. pyogenes* with respect to the structure of the polysaccharide and the capsule synthesis genes; in type 3 pneumococci, capsule synthesis involves three genes that are homologs of *hasABC* (31, 32). In both *S. pyogenes* and type 3 pneumococci, it seems likely that the capsule synthesis gene cluster was acquired from another species by horizontal gene transfer. This hypothesis is also suggested by genome comparisons of *S. pyogenes* with group B *Streptococcus*: with the exception of the *hasABC* cluster that is absent in group B *Streptococcus*, there is a high degree of gene synteny over more than 25 kb of the corresponding chromosomal region in *S. pyogenes* and group B *Streptococcus*.

While the *has* operon is highly conserved, it is not universally present in *S. pyogenes* clinical isolates. In particular, strains of M types 4 and 22 and some isolates of M 89 lack the capsule synthesis operon and do not produce a hyaluronic acid capsule (33–36). Interestingly, the absence of the capsule synthesis genes in M types 4 and 22 is associated with the presence of enzymatically

active hyaluronidase, whereas in serotypes that produce capsule, the chromosomal *hylaA* gene contains a point mutation that renders it catalytically inactive. These divergent patterns of gene expression suggest that *S. pyogenes* has evolved two mutually exclusive survival strategies: production of an antiphagocytic hyaluronate capsule or production of hyaluronidase, presumably as a spreading factor in host tissues and/or to degrade host hyaluronic acid as a source of nutrition.

Regulation of Hyaluronic Acid Biosynthesis

The *has* operon is highly conserved among *S. pyogenes* strains. A study of nine *S. pyogenes* isolates that came from diverse settings and whose dates of isolation spanned 75 years found only six mutations within a 300-nucleotide central region of the *hasA* gene (17); all were single nucleotide substitutions, and none resulted in a change in the amino acid sequence of the protein. Despite the high degree of conservation of the hyaluronan synthase gene, individual *S. pyogenes* strains vary widely in the amount of capsule they produce. Since the *has* genes are conserved, differences in capsule expression among strains are likely to reflect differences in the regulation of *has* gene transcription. Experimental support for this hypothesis comes from the observation that *has* gene transcripts are detected during exponential growth of

well-encapsulated strains of *S. pyogenes* but not in strains that do not produce detectable hyaluronic acid (24). Analysis of the *has* operon promoter using a reporter system in which the promoter was fused to a promoterless chloramphenicol acetyl transferase (CAT) gene showed that full activity of the *has* promoter required no more than 12 nucleotides of chromosomal DNA upstream of the promoter's -35 site (37). This observation is consistent with the finding that many strains of *S. pyogenes* (10 of 23) contain the insertion element IS1239' approximately 50 nucleotides upstream of the -35 site without any apparent effect on capsule production (Figure 2) (23).

One factor that influences the amount of capsule produced by a particular strain is the structure of the *has* operon promoter itself. Experiments using CAT reporter gene fusions revealed 3-fold higher activity of the *has* promoter from a highly encapsulated M type 18 strain than that from a poorly encapsulated M type 3 strain (37). Construction of hybrid promoters by site-directed mutagenesis demonstrated that nucleotide substitutions in two regions accounted for the differences in promoter activity between the two serotypes: three nucleotides in the -35, -10 spacer region and four nucleotides in the +2 to +8 positions relative to the start site of *hasA* transcription. The characteristic fine structure of the *has* operon promoter in type 18 strains may account in part for the observation that M18 strains are typically mucoid (i.e., highly encapsulated) since the precise sequence of the *has* promoter appears to be serotype-specific.

Capsule production varies not only among strains but also in an individual strain under different circumstances. For example, during growth in liquid batch culture, *S. pyogenes* organisms produce large capsules during the exponential phase and then shed their capsules into the medium during the stationary phase. Studies using a *has* promoter-CAT fusion integrated into the *S. pyogenes* chromosome demonstrated a rapid increase in promoter activity to a maximal level during the exponential phase of growth, with a sharp fall as the organisms entered the stationary phase. The production of cell-associated capsular polysaccharide followed an

identical but slightly delayed pattern, consistent with the expected lag for changes in the translation of enzyme proteins that are subsequently reflected in the rate of polysaccharide synthesis. These results indicate that the changing levels of encapsulation observed during different phases of growth are due to transcriptional regulation of *has* operon expression. Further studies of the effect of growth rate on capsule expression in a chemostat continuous culture system found that levels of CAT activity and capsule production were at least three times higher in cells maintained at a constant rapid growth rate (doubling time of 1.4 hours) than in cells maintained at a slow growth rate (doubling time of 11 hours) (S. Albertí, J.L. Levin, and M.R. Wessels, unpublished observations).

Upregulation of capsule production is likely to occur during infection when the organism moves from the external environment into the pharynx or deep tissues, where both temperature and nutrient availability are optimal for bacterial growth. This hypothesis was supported by experiments using a reporter strain of *S. pyogenes* in which expression of green fluorescent protein was controlled by the *has* operon promoter. Introduction of the strain into the mouse peritoneum or into the baboon pharynx triggered a rapid increase in fluorescence, reflecting transcription from the *has* promoter (38). Thus, the regulation of capsule expression may be a useful adaptation to survival in host environments in which the capsule is required to defend the organism against complement-mediated phagocytic killing.

The dynamic changes in *S. pyogenes* capsule expression with changes in growth rate and the observation that some strains of *S. pyogenes* appear more highly encapsulated after animal passage have suggested that capsule gene transcription is regulated by cellular mechanisms in addition to those attributable to the intrinsic structure of the *has* operon promoter. Mga, the *trans*-acting regulatory protein that influences expression of several *S. pyogenes* proteins, appears not to regulate capsule expression, at least in most strains of *S. pyogenes*. Inactivation of Mga was associated with reduced

FIGURE 2 Map of the region of the *S. pyogenes* chromosome that includes the *has* operon encoding enzymes required for hyaluronic acid synthesis. The other genes shown appear not to be involved in capsular polysaccharide synthesis or surface expression. In some strains insertion sequence IS1239' is present approximately 50 nucleotides upstream of the *has* operon promoter (adapted from the genome sequence of M1 strain SF370, GenBank accession number AE004092 [93]).



has gene transcription in an M1 strain (39); however, no effect on capsule production and/or *has* transcription was observed upon Mga inactivation in strains of M6, M18, or M49 (40–42). RofA, a regulatory protein involved in control of protein F expression, also is not known to affect capsule production (43).

Transposon mutagenesis of a poorly encapsulated M3 strain of *S. pyogenes* resulted in the identification of a novel regulatory locus whose inactivation dramatically increased capsule production (42). The locus consists of two genes, *csrR* and *csrS* (also called *covR* and *covS*), whose predicted products resemble the regulator and sensor proteins, respectively, of bacterial two-component regulatory systems (Fig. 3). Targeted inactivation of *csrR* by allelic exchange mutagenesis resulted in a 6-fold increase in capsule production. The increase in hyaluronic acid synthesis was accompanied by a parallel increase in *has* gene transcription—evidence that CsrR regulates transcription of the capsule synthesis genes. Mutation of *csrR* appeared not to affect expression of M protein or hemolytic activity, but subsequent work in several laboratories has demonstrated that the system also represses expression of several other virulence factors, including streptokinase (*ska*), streptolysin S (*sag* operon), mitogenic factor/streptodornase (*speMF*), an integrin-like protein/IgG protease (*mac* or *ideS*), and in some strains, cysteine protease (*speB*) (44–47). Microarray transcriptional profiling experiments suggested that CsrRS is involved directly or indirectly in regulation of approximately 10% of *S. pyogenes* genes (44, 48–50). The membrane-associated CsrS protein is a histidine kinase thought to respond to at least two environmental signals: an elevated extracellular Mg^{2+} concentration is associated with increased phosphorylation of the cog-

nate regulator CsrR, presumably as a result of stimulating CsrS autokinase activity and/or inhibiting CsrS phosphatase activity for CsrR (51). The human antimicrobial peptide LL-37, at concentrations below those that inhibit *S. pyogenes* growth, has an effect on CsrR phosphorylation opposite to that of elevated magnesium (52, 53). Phosphorylation of CsrR increases its affinity for binding to the promoter region of the *has* operon and most other regulated genes to repress transcription (46, 54, 55). The likely importance of this regulatory system in virulence is supported by the markedly greater resistance to *in vitro* phagocytosis displayed by a (highly encapsulated) *csrR* mutant than by the wild-type parent strain and a 500-fold increase in the mutant's virulence for mice after intraperitoneal challenge (42). Up to 40% of invasive *S. pyogenes* isolates have inactivating mutations in CsrS or CsrR, whereas such mutations are rare among isolates from pharyngitis patients (56–58).

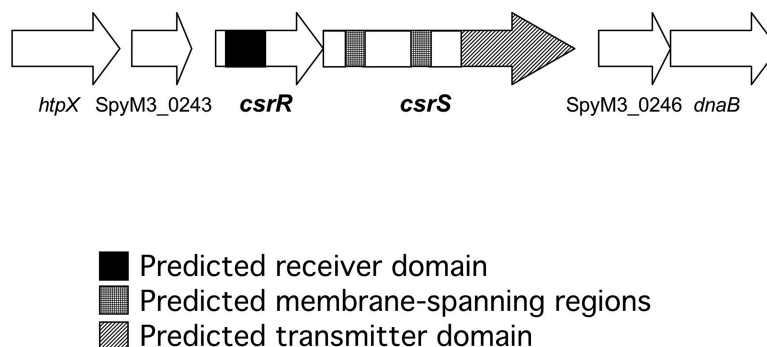
Capsule production is also controlled by RocA, a protein first identified as a regulator of CsrR expression and later shown to influence phosphorylation of CsrR (59, 60). Mutations in *rocA* are associated with increased expression of the *has* operon and of other genes regulated by the CsrRS system. *S. pyogenes* strains of *emm* types 18 and 3 each harbor a mutation in *rocA* that results in expression of a truncated RocA protein and increased capsule production (61, 62).

THE CAPSULE AND HOST IMMUNE DEFENSES

Immunogenicity of Hyaluronic Acid

In 1937, Kendall et al. reported the purification of capsular polysaccharide from mucoid strains of *S. pyogenes*

FIGURE 3 Diagram of the *csrRS* chromosomal locus encoding a two-component regulatory system that regulates hyaluronic acid synthesis. Sequences corresponding to regions of the predicted proteins with properties characteristic of the response regulator (CsrR) or sensor (CsrS) components are indicated (adapted from the genome sequence of M3 strain MGAS315, GenBank accession number AE014074 [94]).



(63). Their analysis revealed that an apparently identical capsular material was produced by different M types of *S. pyogenes*. The *S. pyogenes* polysaccharide contained equal amounts of *N*-acetylglucosamine and glucuronic acid as exclusive components of monosaccharides; this finding suggested that it might be structurally identical to a polysaccharide isolated from bovine vitreous humor and human umbilical cord. Immunization of rabbits and horses with mucoid *S. pyogenes* cells failed to elicit antibodies detectable by precipitin reactions with the polysaccharide. Later studies have confirmed that the “serologically inactive” polysaccharide produced by *S. pyogenes* is hyaluronic acid, a high *M*_n polysaccharide with a repeating unit structure identical to that of hyaluronic acid isolated from animal sources. Presumably because it is recognized as a “self” antigen, hyaluronic acid is poorly immunogenic in several animal species. Fillit and colleagues evoked antibodies to hyaluronic acid by immunization of rabbits with encapsulated streptococci of group A or C and by immunization of mice with hyaluronidase-treated hyaluronic acid linked to liposomes (64, 65). Such antibodies, however, have not been shown to have any opsonic activity against *S. pyogenes* *in vitro* or to confer protection against *S. pyogenes* infection *in vivo*.

Capsule and Resistance to Phagocytosis

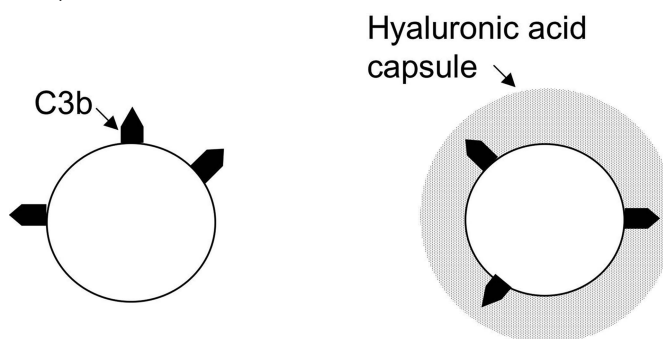
S. pyogenes, like other Gram-positive pathogens, is resistant to direct bacteriolysis by complement and antibodies. Therefore, clearance of the organisms from the blood or deep tissues depends largely on uptake and killing by phagocytes. This concept was supported by the observations of Todd, Lancefield, and others that virulence of *S. pyogenes* isolates in both clinical settings and in experimental animal infections was closely paralleled by the capacity of the strains to resist killing by human blood leukocytes. *S. pyogenes* strains that produced large amounts of M protein multiplied in fresh human blood, while those that lacked M protein did not (66, 67). In addition, however, early studies demonstrated a relationship between surface expression of the hyaluronic acid capsule and resistance to phagocytosis. Mucoid or highly encapsulated strains tended to be resistant, and hyaluronidase treatment increased their susceptibility to phagocytosis *in vitro*, a result that suggested a protective effect of the capsule (68–70). More recent work with acapsular mutants has provided definitive evidence that the capsule is a major determinant of resistance to phagocytic killing. In several assays measuring complement-dependent phagocytic killing by human blood leukocytes, acapsular mutants of type 18

and type 24 *S. pyogenes* were significantly more susceptible than the corresponding encapsulated wild-type strains (14, 71, 72).

One mechanism through which capsular polysaccharides may confer resistance to phagocytosis is the inhibition of complement activation. The type III capsular polysaccharide of group B *Streptococcus*, for example, prevents deposition of C3b on the bacterial surface through inhibition of alternative complement pathway activation by sialic acid residues present in the polysaccharide (73, 74). In *S. pyogenes*, however, the presence of the capsular polysaccharide appears to have no effect on the amount of C3b deposited on the bacterial surface. Similar amounts of C3 were detected on acapsular mutants and on encapsulated wild-type strains after incubation of the organisms in serum (71). The increased resistance of well-encapsulated *S. pyogenes* strains to complement-dependent phagocytic killing, therefore, must reflect the capacity of the capsule to block access of neutrophils to opsonic complement components bound to the bacterial surface rather than inhibition of C3b deposition (Fig. 4).

Resistance of *S. pyogenes* to phagocytosis has been assessed most often with the bactericidal test of Lancefield, in which a small inoculum of the test strain is rotated for 3 hours in heparinized fresh human blood (67). Strains that exhibit a significant increase in CFU during rotation in blood are considered resistant. An effect of the capsule is evident in this assay (acapsular mutants show less net growth than encapsulated wild-type strains), but the effect of M protein is more striking (14, 72). M protein-deficient strains not only fail to grow but typically are reduced in number after a 3-hour incubation. By contrast, when resistance to phagocytosis is assessed with a different assay, the effect of capsule is

FIGURE 4 The hyaluronic acid capsule and resistance to complement-mediated phagocytosis. The capsule does not prevent deposition of C3b on the bacterial cell wall but, rather, interferes with the interaction of bound C3b with phagocyte receptors.



more dramatic than that of M protein. In this second assay system, a larger bacterial inoculum is rotated for 1 hour with human peripheral blood leukocytes in the presence of 10% normal human serum as a complement source (75, 76). In the latter assay, acapsular mutant strains and poorly encapsulated wild-type strains are killed (~90% reduction in CFU) despite the presence of M protein, while well-encapsulated strains do not change or increase in number. A mutant that is deficient in M protein but that produces wild-type amounts of capsule is killed less efficiently than a capsule-deficient mutant in the same background (72). In a study of isogenic mutants deficient in capsule, M protein, or both in the background of a mucoid type 18 *S. pyogenes* strain, mouse virulence correlated better with results in the second assay system than with the Lancefield whole-blood assay (72).

CAPSULE AS A VIRULENCE FACTOR

Experimental Infection Models

In the 1940s, investigators attempted to determine experimentally whether the hyaluronic acid capsule contributed to the virulence of *S. pyogenes*. Hirst found that decapsulating *S. pyogenes* by treatment with leech extract containing hyaluronidase had no effect on virulence of the bacteria in mice (77). However, Kass and Seastone found reduced virulence of decapsulated *S. pyogenes* in mice, perhaps because the latter authors administered hyaluronidase to the infected mice at frequent intervals to prevent regeneration of the capsule after challenge (78). Subsequently, more direct proof that the capsule contributes to virulence has come from several studies using acapsular mutant strains of *S. pyogenes* derived by transposon mutagenesis or by targeted inactivation of gene(s) required for hyaluronic acid synthesis (Table 1).

Because the capsule is required for *S. pyogenes* resistance to complement-mediated phagocytic killing (see

above), it is not surprising that capsule expression enhances virulence in systemic infection models in which the animals succumb to overwhelming bacteremia. In addition, however, the hyaluronic acid capsule contributes in a pivotal fashion to the capacity of the organisms to produce invasive soft tissue infection. A mouse model of invasive *S. pyogenes* soft tissue infection has been developed in which the bacterial inoculum is delivered by superficial injection just below the skin surface in a small volume and with minimal trauma to the tissues (79, 80). Mice challenged with the highly encapsulated M24 strain Vaughn developed dermal necrosis with underlying purulent inflammation and secondary bacteremia, while mice challenged with the acapsular mutant strain 24-4 developed no lesion at all or minor superficial inflammation and no bacteremia (79). Similar results were observed in the same model with a moderately encapsulated M3 strain of *S. pyogenes* (originally isolated from a patient with necrotizing fasciitis) which was compared with two isogenic acapsular mutants derived by allelic exchange mutagenesis of the *hasA* (hyaluronan synthase) gene. In the latter experiments, the majority of animals challenged with the encapsulated M3 parent strain developed spreading, necrotic soft tissue infection and died, whereas the acapsular mutants produced only mild local infection and no deaths (80). The dramatic impact of the capsule on virulence in this model is correlated with equally striking histopathologic findings: In animals challenged with an acapsular mutant, a small focus of neutrophilic inflammation and necrosis is confined by a well-formed abscess. By contrast, after challenge with the encapsulated wild-type strain, acute inflammatory cells extend throughout the subcutaneous tissue and are associated with thrombosis of blood vessels, with infarction and necrosis of the overlying dermis—a histopathologic picture very similar to that in patients with *S. pyogenes* necrotizing fasciitis. Examination of the histologic sections by immunofluorescence microscopy with an *S. pyogenes*-specific antibody showed that the acapsular *S. pyogenes* cells were confined to the abscess cavity, while the encapsulated wild-type organisms were widely dispersed throughout the subcutaneous tissues.

Capsule and Mucosal Colonization

Studies by at least two groups have demonstrated a critical role for the hyaluronic acid capsule in pharyngeal colonization in mice. BALB/c mice inoculated intranasally with the stable (transposon insertion plus chromosomal deletion) acapsular mutant strain 24-4 rapidly cleared the organism from the pharynx, and no mice

TABLE 1 Animal models in which the hyaluronic acid capsule has been shown to enhance virulence of group A streptococci

Type of infection	Animal species	Reference(s)
Systemic infection	Mouse	14, 76
	Chicken (embryo)	95
Pharyngeal colonization	Mouse	81, 82
	Baboon	83
Pneumonia	Mouse	82
Soft tissue infection	Mouse	79, 80

died (81). In contrast, throat cultures of mice challenged with the revertible (simple transposon insertion) acapsular mutant 24-72 yielded encapsulated revertants, and the rate of persistent throat colonization and lethal systemic infection was similar to that in animals challenged with the encapsulated type 24 parent strain. A study of an acapsular mutant derived from the mouse-virulent M50 strain, B514, yielded similar results: intranasal challenge with the wild-type strain resulted in chronic throat colonization in C57BL/10SnJ mice. After intranasal challenge with an acapsular mutant derived by plasmid insertion mutagenesis of the *hasA* gene, encapsulated revertants accounted for all *S. pyogenes* recovered from throat cultures 4 days after challenge (82). Intratracheal inoculation of C3HeB/FeJ mice with the wild-type strain produced pneumonia and secondary systemic infection in approximately 50% of the animals within 72 hours, while only 2 animals challenged with the acapsular mutant became ill, 5 and 6 days after challenge, respectively. In both cases only encapsulated revertants were recovered from cultures of the throat, lungs, and blood (82). The findings in both studies of impaired colonization by the acapsular mutant strains and the rapid emergence of encapsulated revertants together provide strong evidence that encapsulation confers a powerful survival advantage for *S. pyogenes* in the upper airway. This conclusion was supported also by experiments in a primate model of *S. pyogenes* pharyngeal colonization: an acapsular mutant derived from an M3 strain was cleared from the pharynx of baboons more quickly than the wild-type parent strain (83). While these results suggest that capsule production enhances initial survival and persistence of *S. pyogenes* in the pharynx, studies of long-term carriage in a primate model and in human carriers have shown that carriage isolates acquire mutations that result in reduced or absent hyaluronic acid production (84, 85). Taken together, the results of these studies suggest that the hyaluronic acid capsule enhances transmission and initial colonization but that prolonged carriage in human beings may be favored by subsequent downregulation or loss of capsule production.

Capsule and *S. pyogenes* Attachment to Epithelial Cells

The influence of the hyaluronic acid capsule on the capacity of *S. pyogenes* to colonize the pharyngeal epithelium may be explained, at least in part, by the protective effect of the capsule in preventing opsonophagocytic killing, although the importance of resistance to opsonophagocytosis at a mucosal site is less well-defined than in

blood or deep tissues. In addition, the capsule is an important factor in the process of *S. pyogenes* attachment to epithelial cells, both by modulating interactions of other bacterial surface molecules with the epithelium and by direct interaction of *S. pyogenes* capsular hyaluronic acid with eukaryotic cells. The capsule may influence adherence by interfering with the interaction of any of a variety of *S. pyogenes* surface molecules that have been implicated as adhesins: M and M-like proteins, fibronectin-binding proteins, lipoteichoic acid, and glyceraldehyde-3-phosphate dehydrogenase. In general, acapsular mutant strains of *S. pyogenes* attach in higher numbers to skin or throat keratinocytes than do the corresponding encapsulated wild-type strains. For type 24 *S. pyogenes*, the capsule prevents M protein-mediated adherence to keratinocytes, since inactivation of the *emm24* gene reduced adherence in the background of an acapsular mutant but not in the background of the same strain expressing wild-type amounts of capsule (86). It is likely that the capsule exerts a similar modulating influence on bacterial adherence mediated by other *S. pyogenes* surface molecules.

Not only does the capsule influence the binding interactions of other *S. pyogenes* surface structures, but there is evidence that the capsule itself can serve as a ligand for attachment of *S. pyogenes* to CD44 on epithelial cells. CD44 is a hyaluronic acid-binding glycoprotein found on a variety of human cells, including epithelial and hematopoietic cells. The interaction of CD44 with hyaluronic acid appears to have important functions in cell migration, organization of epithelia, lymphocyte homing, and tumor metastasis. Because CD44 is expressed on the surface of tonsillar epithelial cells, it may serve as a receptor for binding of hyaluronic acid on the surface of *S. pyogenes*. This hypothesis was supported by the finding that a monoclonal antibody to CD44 reduced attachment of wild-type strains of *S. pyogenes* to human pharyngeal keratinocytes by ~90% but had no effect on attachment of acapsular mutant strains (86). Further evidence that CD44 acts as a receptor for *S. pyogenes* came from the observation that transfection of K562 cells with cDNA encoding either of two epithelial isoforms of CD44 conferred on the cells the capacity to bind *S. pyogenes*. Cells expressing CD44 bound a variety of *S. pyogenes* wild-type strains, including several in which the level of capsule expression was below the limit of detection in a dye-binding assay; however, inactivation of the hyaluronan synthase gene in such a poorly encapsulated *S. pyogenes* strain completely abrogated binding (86). A study of transgenic mice with defective expression of epithelial CD44 found

reduced pharyngeal colonization compared to that in wild type mice after intranasal challenge with *S. pyogenes*, evidence that CD44 can function as a receptor for *S. pyogenes* binding to the pharyngeal epithelium *in vivo* (87).

Capsule and CD44-Mediated Tissue Invasion

CD44 is a transmembrane protein, and its ligation by hyaluronic acid can result in signal transduction and induction of cellular events involved in cell migration. Binding of *S. pyogenes* to CD44 on human pharyngeal keratinocytes *in vitro* has been shown to trigger a similar pattern of cellular responses, including the formation of lamellipodia or membrane ruffles at the site of *S. pyogenes* attachment and disruption of intercellular junctions (88). These events were associated with enhanced translocation of encapsulated *S. pyogenes* compared to an acapsular mutant across epithelial cell layers *in vitro*. The results suggested that interaction of the *S. pyogenes* capsule with CD44 manipulates a host signaling pathway to disrupt epithelial integrity and enable bacterial penetration into underlying tissues.

Capsule and *S. pyogenes* Entry into Epithelial Cells

Several laboratories have documented the entry of *S. pyogenes* into human epithelial cells, a process that has been proposed to represent a virulence mechanism—either a step in bacterial invasion into deep tissues or a means of escape of the organism into an intracellular sanctuary shielded from antibiotics and host immune defenses (79, 89–91). However, while there is irrefutable evidence that the capsule plays a key role in invasive infection *in vivo*, there is equally clear evidence that the capsule prevents *S. pyogenes* entry into epithelial cells. In studies of *S. pyogenes* entry into cultured human keratinocytes from pharyngeal epithelium or external skin, poorly encapsulated strains entered cells far more efficiently than well-encapsulated strains, regardless of M type or clinical source of the isolates (79). Acapsular mutants entered more than 1,000 times more efficiently than corresponding encapsulated parent strains. The apparently opposite effects of the capsule on invasive infection *in vivo* compared to those on epithelial cell “invasion” *in vitro* suggest that bacterial entry into epithelial cells is not a step in productive infection but rather a host response that limits the spread of poorly encapsulated strains. By contrast, virulent encapsulated strains resist ingestion by eukaryotic cells, both epithelial cells and professional phagocytes, and invade deep tissues by an extracellular route, a process that appears to

be facilitated by CD44-mediated disruption of cell-cell junctions. This hypothesis is supported by the finding that clinical isolates from patients with invasive *S. pyogenes* infection “invade” epithelial cells at much lower rates than throat or skin isolates (92). After internalization, the viability of *S. pyogenes* within epithelial cells *in vitro* falls rapidly after bacterial entry (79, 90). It is possible, however, that transient intracellular survival of *S. pyogenes*, perhaps in a cyclic fashion, contributes to persistent carriage.

CONCLUSIONS

Careful observations by microbiologists and clinicians in the 1920s and 1930s pointed to a link between the hyaluronic acid capsule and *S. pyogenes* disease pathogenesis. More recent studies have defined the genetic locus that directs hyaluronic acid biosynthesis and have characterized the molecular mechanisms through which the capsule enhances *S. pyogenes* virulence. It is now appreciated that the *has* capsule synthesis operon is both highly conserved and widely, but not universally, distributed among *S. pyogenes* strains, attesting to the adaptive role played by the hyaluronic acid capsule in the coevolution of *S. pyogenes* with the human host. As a poorly immunogenic “self” antigen, the capsular polysaccharide appears to have persisted in an invariant form in *S. pyogenes*, presumably because it has not been subject to the selective pressure of an effective, polysaccharide-specific, humoral immune response in the infected host that has led to emergence of multiple capsular types in other pathogenic bacteria. Studies of acapsular mutant strains have demonstrated that the capsule protects *S. pyogenes* from complement-mediated phagocytic killing and is essential for full virulence in a variety of experimental infection models. The *S. pyogenes* capsule also influences attachment of the bacteria to human epithelial cells, both by modulating the interaction of M protein and other potential adhesins and by itself serving as a ligand for attachment of *S. pyogenes* to CD44 on epithelial cells. The latter mechanism may be of particular importance in *S. pyogenes* colonization of the pharynx, because it appears to participate in the adherence of diverse *S. pyogenes* strains, including those that produce only small amounts of capsule. Finally, the successful adaptation of *S. pyogenes* to survival in the human host involves regulation of capsule expression that is dependent on the fine structure of the *has* operon promoter, on the CsrRS two-component regulatory system, and likely, on additional mechanisms yet to be uncovered.

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